American Journal of Hematology 53:221–227 (1996)

Congenital 6-Phosphogluconate Dehydrogenase (6PGD) Deficiency Associated With Chronic Hemolytic Anemia in a Spanish Family

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Clinical and metabolic studies were performed in four members of a Spanish family with partial (50%) 6 phosphogluconate dehydrogenase (6PGD) deficiency. In all cases the activities of 6 phosphogluconolactone (6PGL) and glutathione reductase (GR) were normal, and the molecular characterization performed in the partially purified 6PGD from the propositus showed normal kinetic and electrophoretic patterns. Two females (the propositus and her sister) suffered from a well-compensated chronic nonspherocytic hemolytic anemia (CNSHA) and exhibited decreased RBC glutathione (GSH) stability with increased oxidative susceptibility, defined by enhanced malonyldialdehyde (MDA) generation "in vitro." The other two members of the family (the propositus's mother and brother) were clinically asymptomatic. In the propositus and her sister, RBC metabolism exhibited a markedly abnormal concentration of glycolytic intermediates, mainly characterized by striking increases in fructose 1,6 bisphosphate (50-fold), dihydroxiacetonephosphate (20-fold) and glyceraldehyde 3-phosphate (tenfold).

Although the precise mechanism of the hemolysis in the two patients is unknown, the enhanced oxidative threat observed in their RBCs may interfere in some way with the glycolytic pathway function, leading to a marked increase in certain metabolic intermediates located before the glyceraldehyde 3 phosphate dehydrogenase (GA3PD) step. Since it seems that GA3PD half-life is modulated by fluctuations of the cytosolic redox status, an "in situ" approach was simulated by using permeabilized RBCs. In these conditions, GA3PD activity was significantly lower in the propositus and her sister than in the asymptomatic members of the family and the simultaneous normal control. © 1996 Wiley-Liss, Inc.

Key words: 6 phosphogluconate dehydrogenase, 6PGD, hemolytic anemia, red cell enzyme deficiency

INTRODUCTION

6 phosphogluconate dehydrogenase (E.C. 1.1.1.44, 6PGD) is a polymorphic enzyme in the hexose monophosphate shunt (HMPS) that catalyzes the conversion of 6-phosphogluconate to ribulose 5-phosphate. 6PGD and glucose-6-phosphate dehydrogenase (EC 1.1.1.49;G6PD) are the key enzymes in the HMPS, and while G6PD is the most common enzymopathy in humans [1–3], 6PGD deficiency is extremely rare and its coexistence with slight hemolysis has been reported in only two cases [4,5]. From a genetical point of view, there appear to be at least two distinct types of 6PGD gene mutations; one resulting in approximately 50% of normal enzyme activity in heterozygotes, and the other, associated with approximately 75% of activity in heterozygotes, results in almost total deficiency in homozygotes [6,7].

Clinical and metabolic studies were performed in four members of a Spanish family with heterozygous 6PGD deficiency. Two of them (the propositus and her sister) suffered from chronic nonspherocytic hemolytic anemia

Received for publication January 19, 1996; accepted June 18, 1996.

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(CNSHA) and the other two were clinically asymptomatic. In the two patients with CNSHA, striking increases in fructose 1,6 bisphosphate and some other glycolytic intermediate abnormalities before the glyceraldehyde-3-phosphate dehydrogenase (GA3PD) step were also observed. These abnormalities were not present in the asymptomatic members of the family (mother and brother). The permeabilization of RBCs for "in situ" studies of intracellular glycolytic enzymes [8] revealed a substantial decrease of GA3PD activity only in the clinically affected members. This suggests that a metabolic block at the GA3PD level may account for the abnormal glycolytic intermediate pattern observed in the patients. However, the relationship between the partial 6PGD deficiency and this metabolic abnormality, if it exists, remains to be demonstrated.

PATIENTS AND METHODS

Case Report

The propositus was 26 years old and she came to our laboratory after diagnosis of chronic hemolytic anemia. At the time of the study she exhibited a slight anemia (hemoglobin of 116 g/L) and moderate reticulocytosis (4.1%). The study of hemolysis revealed a partial 6PGD deficiency (40–60% of normal activity) in RBCs and leukocytes and a slight decrease of glucose-6-phosphate dehydrogenase (G6PD) activity. The family study demonstrated the existence of 6PGD deficiency but not of G6PD in the propositus's mother, sister, and brother. Whereas the mother and brother were clinically and hematologically normal, the sister also showed a well-compensated hemolytic anemia (hemoglobin of 113 g/L and reticulocyte count of 3.0%).

General Hematological Studies and In Vitro Assay of RBC Peroxidation

Routine hematological studies were performed using standard laboratory methods. Leukocytes were removed from heparinized blood by filtration through microcrystalline cellulose and α-cellulose [9] and then hemolyzed with a lysing solution (EDTA 0.27 mM, pH 7.0, and 2-mercaptoethanol 0.05%) and kept at −80°C until use. Malonyldialdehyde (MDA) production by RBCs, a measure of polyunsaturated fatty acid (PUFA) oxidation, was determined following Stocks and Dormandy [10]. Briefly, RBCs from normal and patient's blood free of antioxidants or preservatives, were washed twice in isosmotic phosphate-saline buffer and the final packed cell volume (PCV) adjusted to precisely 5% by hemoglobin estimations. RBC suspension (2 mL) was incubated with an equal volume of 20 mM H_2O_2 solution for 60 min at 37°C and MDA production (nmol/gHb) was measured with a Kontron® spectrophotometer.

RBC Metabolic Studies and Permeabilization of Erythrocytes for the "In Situ" Assay of Glyceraldehyde-Phosphate Dehydrogenase (GA3PD)

Red cell enzymes (glycolytic, nucleotidic, and glutathione metabolism) were measured as described by ICSH [11] and 6-phosphogluconolactonase (6PGL) following the method of Beutler et al. [12]. Reduced glutathione (GSH) and GSH stability test were performed following Beutler et al. [13]. Glycolytic intermediates were studied as described in [14]. Assay for 2,3-bisphosphoglycerate (2,3-BPG) was performed by the Sigma (St. Louis, MO) kit. Fructose 2,6-bisphosphate (fructose 2,6-P_2) and its synthesizing enzyme 6-phosphofructo-2-kinase (PFK-2) were performed in accordance with the method of Van Schaftingen et al. [15] with some modifications for the erythrocyte measurement [16]. For "in situ" measurement of GA3PD, washed RBCs were treated with dimethyl suberimidate dihydrochloride (DMS) as described by Aragon et al. [8] and Aragon and Sols [17], with slight modifications. Briefly, 40 g of DMS was dissolved in 7 ml of 75 mM NaCl, 100 mM triethanolamine pH 8.7, and the solution was adjusted to pH 8.5 with 0.1 M NaOH and mixed immediately with 2 ml of washed erythrocytes. GA3PD activity was determined using different concentrations of NAD (10 to 0.05 mM). Normal values are obtained from 50 normal controls and the reference values are expressed using two standard deviation.

Molecular and Kinetic Studies

Molecular study of the 6PGD enzyme was performed on partially purified enzyme [18]. RBCs were washed with isotonic saline solution and lysed with 1.5 mM sodium phosphate buffer. The hemoglobin was removed using DEAE Sephadex A-50 and the partially purified enzyme was obtained by precipitation with ammonium sulfate. The precipitate was dissolved in 10 mM Tris-HCl buffer, pH 7.5, and was kept at −20°C with the addition of glycerol or directly used after dialysis against 10 mM Tris-HCl, pH 7.5. Peripheral blood leukocytes were obtained by Ficoll sedimentation as described by Boyum [19]. We used the partially purified enzyme to study the following kinetic parameters: (1) 6PGD activity in leukocytes and erythrocytes measured as described by ICSH [11]; (2) Electrophoretic mobility in Tris-EDTA-borate buffer at pH 8.0; (3) Apparent affinity for substrates (Km for 6PG and NADP); (4) Heat stability at 46°C; and (5) pH optimum curve.

Analysis of G6PD Gene by Polymerase Chain Reaction and Restriction Enzyme (PCR-RE) and PCR-Single Strand Conformation Polymorphism (SSCP)

Genomic DNA was extracted from peripheral blood leukocytes from the affected individual. The DNA region

TABLE I. General Hematological Data of the Patient and Her Family

	Propositus	Sister	Mother	Brother	Reference values
RBC count ($\times 10^{12}$/L)	3.6	3.5	4.5	5.3	4.1–9.9
Packed cell volume (L/L)	37	35	39	42	35–44
Hemoglobin (g/L)	116	113	127	146	120–144
MCV (fL)	102	101	85	80	85–95
MCHC (g/L)	315	321	318	348	303–352
Reticulocytes (%)	4.1	3.0	0.7	0.6	0.2–2.0
WBC count ($\times 10^9$/L)	5.5	5.5	8.8	4.7	5.5–9.6
Platelet count ($\times 10^9$/L)	289	337	250	320	140–350
MDA production by RBCs (nM/g Hb)	518	475	236	313	304–450
RBC 51CrT/2 (days)	18	16	23	25	23–25

from the G6PD gene encompassing each point mutation of specific G6PD variants was selectively amplified by PCR using oligonucleotide primers described previously [20] followed by digestion with restriction enzymes that recognize artificially created or naturally occurring restriction sites [21,22]. Because none of the most common mutations were found, the entire coding region of the G6PD gene and partial intron sequences next to the exons was amplified by PCR from genomic DNA in eight fragments, followed by SSCP analysis of each fragment [23]. No shifts in the normal mobility of single-stranded DNA fragments were found, indicating gene normality.

RESULTS

Routine Hematological Data and "In Vitro" MDA Production by Erythrocytes

Routine hematological parameters and data concerning MDA production by erythrocytes of the propositus and her family are summarized in Table I. As shown, the propositus and her sister exhibited signs of a hemolytic anemia and significantly increased values of MDA production by RBCs. General hemolysis tests performed in the propositus ruled out the coexistence of any of the other most frequent mechanisms of congenital hemolytic anemia (hereditary spherocytosis or unstable hemoglobinopathy).

RBC Metabolic Studies and G3PD Activity Measurement in Permeabilized RBCs

The activities of RBC enzymes involved in the HMP shunt and the levels of reduced glutathione (GSH) are summarized in Table II. With the exception of 6PGD, all these enzymes activities were normal. The propositus showed a slight decrease in G6PD activity. Although the concentration of GSH was within the normal range, the two females with chronic hemolysis (propositus and her sister) exhibited marked GSH instability, as shown by its concentration after 2 hr of incubation in the presence of acetylphenylhydrazine (APH). The other RBC enzyme activities of the glycolytic and nucleotidic pathways, also studied in all family members, were within the normal range (Fig. 1). The glycolytic intermediate content of RBCs was abnormal only in the propositus and her sister (Fig. 2). It was mainly characterized by a striking increase in fructose 1,6-bisphosphate (F16BP) (>4,000%), dihydroxiacetone phosphate (DHAP), and glyceraldehyde 3-phosphate (GA3P). The products of the hydrolysis of F16BP by aldolase were also increased. Fructose 6-phosphate(F6P) and ATP, the precursors of F16BP, were normal and decreased, respectively. This metabolic pattern may be due either to a metabolic block at the glyceraldehyde 3 phosphate dehydrogenase (GA3PD) level in the glycolytic pathway or to a marked increase in the activity of the key glycolytic enzyme 6-phosphofructo-1-kinase (PFK-1). This latter possibility was investigated by measuring the powerful activator of PFK-I, fructose 2,6-bisphosphate, and its syntesizing enzyme 6-phosphofructo 2-kinase (PFK-2) (Table III). The GA3PD activity measured inside permeabilized RBCs was significantly lower in the propositus and her sister than in the other members of the family or the normal controls (Fig. 3).

Molecular Studies of 6PGD and G6PD in Patients' RBCs

The electrophoretic and kinetic characteristics of semipurified RBC 6PGD of the propositus, her brother, and some normal controls are shown in Table IV. The 6PGD activity of the other members of the family was also reduced to about 60% of normal both in RBCs and leukocytes. However, no abnormalities were observed in their enzymatic parameters such as electrophoretic mobility, Km for 6PG and NADP, heat-stability, and pH curve in the semipurified 6PGD enzyme.

The possibility of a heterozygous condition for G6PD deficiency in the propositus was ruled out by DNA analysis. A DNA sample from the patient was first tested for the most frequent G6PD mutations encountered in the Spanish population (G6PD A-$^{376G/202A}$, G6PD Mediterranean^{563T}, G6PD Seattle^{844C}, and G6PD Union^{1360T}); all were negative. SSCP analysis revealed an abnormal mobility of a PCR-amplified DNA fragment encompassing exons XI, XII, and XIII. This was due to the presence of two

TABLE II. RBC Enzymes Related With the Pentose Monophosphate Shunt and RBC Glutathione*

	G6PD	6PGD	6PGL	GR	GR + FAD	GP	GSH*	GSH + APH[a]
Propositus	5.1	4.4	31.6	5.5	9.6	25	80	30
Sister	7.7	3.7	32.5	8.1	15.4	16	79	35
Brother	9.5	4.2	34.2	8.2	10.5	18	56	51
Mother	6.3	3.6	33.6	6.7	10.7	17	68	56
Reference values	5.7–9.9	6.2–10.2	30–38	5.3–12	6.9–15.5	9.1–25	55–83	42–71

*All enzyme activities are expressed as U/g Hb. G6PD: glucose 6-phosphate dehydrogenase; 6PGD: 6-phosphogluconate dehydrogenase; 6PGL: 6-phosphogluconolactonase; GR: glutathione reductase; GP: glutathione peroxidase; GSH: glutathione; APH: acetylphenylhydrazine.
[a]mg/100 ml of erythrocytes.

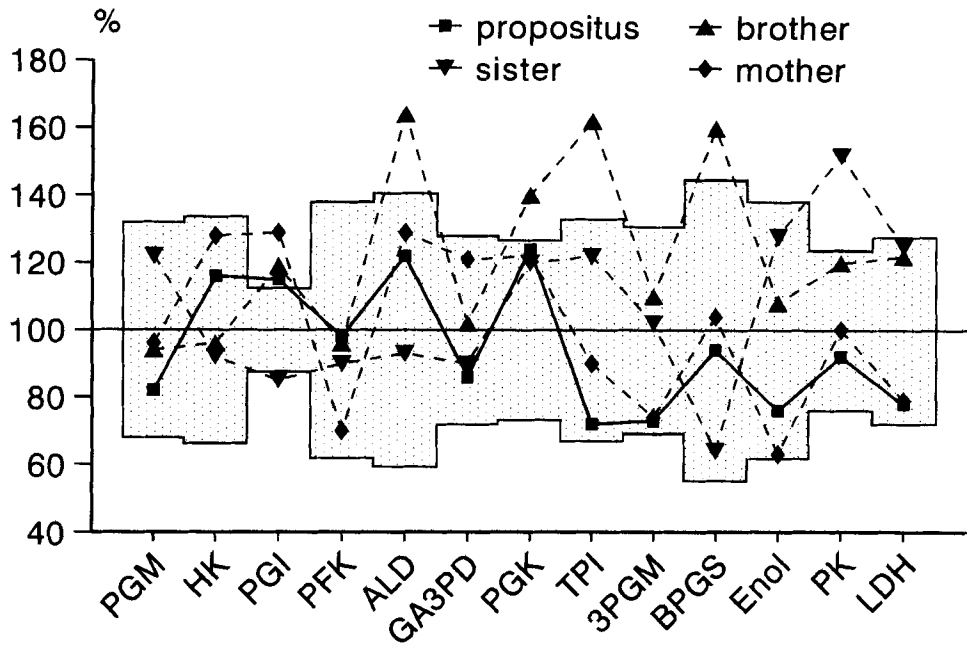

Fig. 1. Graphic representation of RBC enzyme activities in the propositus and her family. Results are expressed in percentages compared with the normal mean (100%) ±SD. PGM: phosphoglucomutase; HK: hexokinase; PGI: phosphoglucose isomerase; PFK: phosphofructokinase; ALD: aldolase; GA3PD: glyceraldehyde 3-phosphate dehydrogenase; PGK: phosphoglycerate kinase; TPI: triose phosphate isomerase; 3PGM: 3-phosphoglyceratemutase; BPGS: bisphosphoglyceratemutase; Enol: enolase; PK: pyruvate kinase; LDH: lactate dehydrogenase.

common silent polymorphic mutations in this fragment: a polymorphic allele in intron 11, as revealed by Nla III digestion as described previously [23] and the 1311 T allele [24,25], confirmed by using Bcl I cleavage after PCR-specific amplification.

DISCUSSION

While G6PD deficiency is the most common RBC enzymopathy associated with hemolytic anemia in humans [1–3], deficiency of the second dehydrogenase in the pentose phosphate shunt, 6PGD, is very rare [26], and has been considered as a "nondisease" by Valentine et al. [27]. The best known cases of 6PGD deficiency are not associated with hemolytic anemia [5,6,28] and only in two French patients a chronic hemolytic anemia has been documented [4,5]. For this reason it is difficult to accept that in such cases this enzyme deficiency is responsible for the hemolysis. In the present report, a woman with well-compensated chronic hemolytic anemia was found to be heterozygous for 6PGD deficiency. The family study displayed the presence of clinical and biological signs of hemolysis only in the patient's sister. The mother and the brother of the propositus, in spite of exhibiting a partial 6PGD deficiency of about 50% in their RBCs and leukocytes, were clinically and hematologically normal. Furthermore, in all cases, RBC enzymatic activities other than 6PGD were normal, including 6-phosphogluconolactonase (6PGL) the deficiency of which, in interaction with a deficiency of G6PD, has been

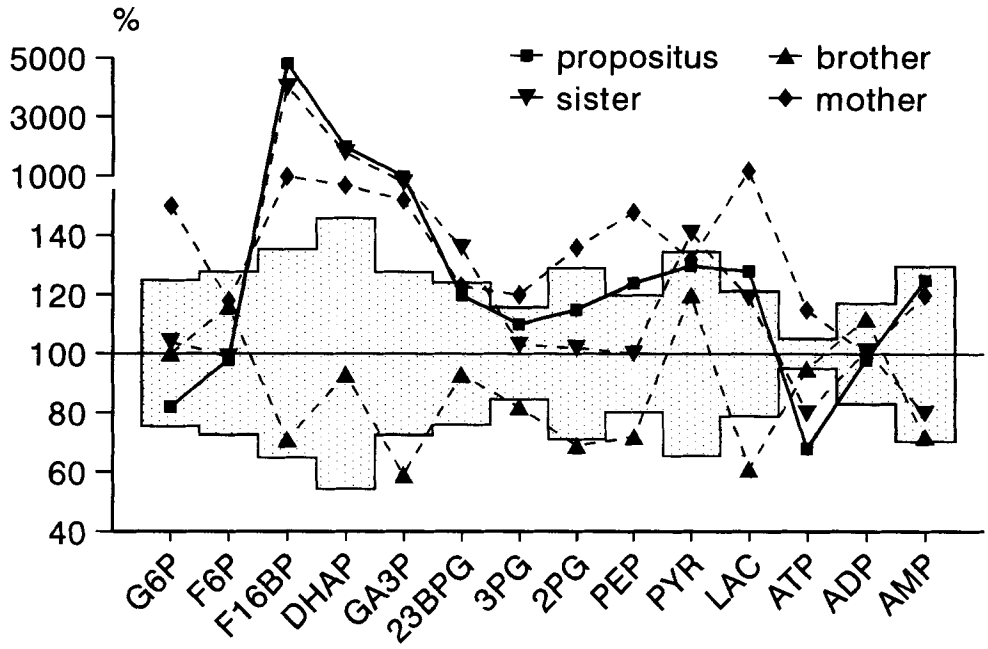

Fig. 2. Graphic representation of RBC glycolytic intermediate compounds in the propositus and her family. Results are expressed in percentages (compared with the normal mean of 100% ±SD). G6P: glucose-6-phosphate; F6P: fructose-6-phosphate; F16BP: fructose-1,6-bisphosphate; DHAP: dihydroxiacetone phosphate; GA3P: glyceraldehyde-3-phosphate; 2,3BPG: 2,3-bisphosphoglycerate; 3PG: 3-phosphoglycerate; 2PG: 2-phosphoglycerate; PEP: phosphoenolpyruvate; PYR: pyruvate; LAC: lactate; ATP: adenosine triphosphate; ADP: adenosine diphosphate; AMP: adenosine monophosphate.

TABLE III. Fructose 2,6-Bisphosphate Levels and 6-Phosphofructo-2-Kinase Activity

	Fructose 2,6-P_2 (pmol/g Hb)	PFK-2 (μU/g Hb)
Propositus	325	42
Sister	364	60
Mother	190	18
Brother	220	20
Normal values	143 ± 38	13 ± 5

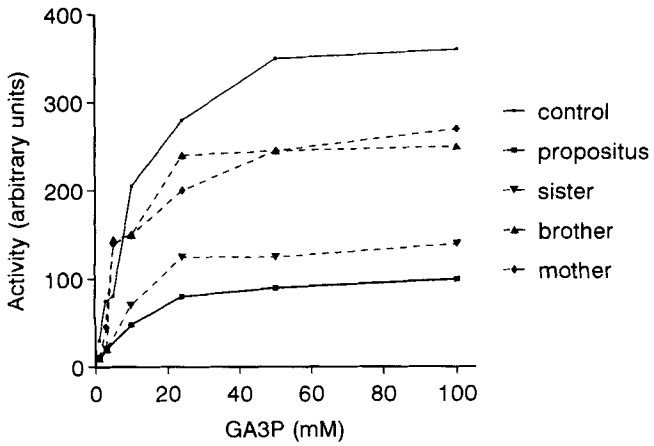

Fig. 3. In situ measurements of glyceraldehyde 3-phosphate dehydrogenase activity using permeabilized RBCs in the propositus, her family, and a normal control. Activity is given in U/g Hb.

considered as a cause of hemolysis [29]. Although no hemolysis are described in one case with combined G6PD and 6PGD deficiency [30], since the propositus exhibited a slightly reduced G6PD activity in RBCs despite the increased number of reticulocytes, a G6PD gene mapping was undertaken in order to test for a possible heterozygous condition for G6PD deficiency. None of the common G6PD variants found in Spain (G6PD A-$^{376G\text{-}202A}$, G6PD Mediterranean^{563T}, G6PD Seattle^{844C}, and G6PD Union^{1360T}) were identified by PCR-RE. Furthermore, after SSCP analysis of the entire G6PD coding gene no PCR-fragments with abnormal mobility were found with the exception of those including exons XI, XII, and XIII characterized by two common polymorphic mutations (1311T and the Nla III site in intron 11) [23–25]. In previous studies, samples containing these known polymorphic mutations failed to show band shift due to another mutation at the same PCR-fragment. This possibility was prevented by dividing the fragment containing exon XI-XII and XIII into shorter fragments before SSCP analysis by two methods: amplifying the target sequence in two overlapping subfragments, one containing exon XI, and the other the exons XII and XIII; and amplifying the fragment in one unit followed by Dde I cleavage. Since the high resolving power of the SSCP technique failed to reveal a mutation, we may assume that no G6PD gene abnormality is present in the patient.

The study of glycolytic intermediates demonstrated an

TABLE IV. 6PGD Activity and Molecular Characteristics of Semipurified Deficient Enzyme

	Propositus	Brother	Reference values
Erythrocyte activity (U/g Hb)	5.3	4.9	6.2–10.2
Leukocyte activity (U/10^9 cells)	10.5	11.9	10.2–24.4
Electrophoretic mobility Tris-EDTA Borate pH 8 (% of normal enzyme)	100	100	100
Apparent affinity (Km)			
Km 6PG (μM)	32	38	31.9–38.9
Km NADP (μM)	10	12.5	8.1–12.3
Optimum pH	8	8	8
Heat stability at 46°C	N	N	N

increase of the ones situated up before the GA3PD step; mainly F16BP which increased, approximately, 40-fold, DHAP, which increased 20-fold, and GA3P which also increased, although less than the others. No aberrant values were observed in other glycolytic intermediates except for a slight decrease in ATP in both patients. A decrease in RBC ATP content, but not in other glycolytic intermediates, was also mentioned in one of the previously reported cases of 6PGD deficiency associated with chronic hemolysis [5]. The mechanism of the striking increase in F16BP is unknown and deserves some comment. The activation of its synthesizing enzyme PFK-1 by fructose 2,6-bisphosphate (fructose 2,6-P_2), a metabolite involved in the control of tissue glucose metabolism [31,32], seems unlikely, since the increases observed in the propositus and her sister may be explained by the young RBC populations (reticulocytes) present in their peripheral blood [16,33]. Therefore, the possibility of a metabolic block of glycolytic pathway at the GA3PD level was investigated. Since red cell GA3PD half life is probably modulated by physiological fluctuations of the cytosolic redox status [34], the simple in vitro assay may be insufficient to characterize the in vivo effectiveness of the enzyme activity. For this reason GA3PD activity was measured in permeabilized RBCs under an in situ approach, which allows better physiological conditions for the study of in vivo behavior of enzymes [5,14]. Our results demonstrated that the in situ GA3PD activity was lower in the 6PGD patients who also exhibit signs of increased RBC oxidative threat (increased MDA production and GSH instability). Therefore, although the different clinical manifestations in members of the same family with the same degree of 6PGD deficiency may be due to differences in the intracellular redox status, the reason for the more severe effect of 6PGD deficiency on the RBC metabolism observed in the propositus and her sister remains unknown.

ACKNOWLEDGMENTS

This work has been partially supported by a grant from the Spanish Ministry of Health (FIS 93/0561). We are also indebted to Prof. Ernest Beutler from the Scripps Research Institute (La Jolla, CA) for his useful suggestions and critical review of the manuscript.

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